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Functional Assignment of the ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 Gene Cluster Involved in the Assembly of Fe-S Clusters in *Escherichia coli*¹

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Fe-S cluster, the nonheme-iron cofactor essential for the activity of many proteins, is incorporated into its target protein by an unknown mechanism. In *Escherichia coli*, genes in the ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 cluster (the *isc* gene cluster) should be involved in the assembly of the Fe-S cluster since its coexpression with the reporter ferredoxin (Fd) dramatically increases the production of holoFd [Nakamura, M., Saeki, K., and Takahashi, Y. (1999) *J. Biochem.* 126, 10-18]. In this study we addressed the functional roles of the proteins encoded by the *isc* gene cluster with respect to the assembly of Fe-S clusters in four reporter Fds. Plasmids were constructed in which eight ORFs in the *isc* gene cluster were individually inactivated either by truncating the coding region or by introducing an oligonucleotide linker containing stop codons. By coexpressing these plasmids with reporter Fds, we show the *iscS*, *iscA*, *hscA*, and *fdx* genes to be required for the assembly of the Fe-S clusters. When these genes were absent from the coexpression plasmid, no overproduction was achieved in any reporter Fds examined. The inactivation of ORF2 and *hscB* had a partial but appreciable effect on the production of some Fds. Deletion of ORF1 produced no difference from the coexpression with the intact *isc* gene cluster. We also examined coexpression using the *fdx* gene in the *isc* gene cluster as a reporter Fd and identified *iscS*, *hscB*, *hscA*, and ORF3 as being involved in the assembly of the [2Fe-2S] cluster in this protein. We propose a model in which the *fdx* gene product functions as an intermediate site for Fe-S cluster assembly.

Key words: expression, ferredoxin, iron-sulfur cluster, iron-sulfur protein, molecular chaperone.

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Proteins containing Fe-S clusters are involved in a number of cellular metabolic processes. The Fe-S clusters function as redox centers in electron transfer reactions, catalytic centers in nonredox enzymes, and sensors of Fe, O₂, and O₂⁻ in the regulation of metabolic pathways (for recent reviews, *1-5*). In Fe-S proteins, iron atoms are bridged by inorganic sulfur atoms in the form of [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters, which are further ligated to the polypeptides by the thiolate side chains of cysteine residues. More complex forms of Fe-S clusters are found in several enzymes, including nitrogenase MoFe-protein (FeMo cofactor and P-cluster) (*6*), iron hydrogenase (H-cluster) (*7, 8*), and sulfite reductase (siroheme-bound [4Fe-4S] cluster) (*9*). Cysteine is the most common ligand to Fe-S clusters in proteins, however, variations are known for some Fe-S proteins including carboxylate ligation in ferredoxin (Fd) from the hyperthermophile *Pyrococcus furiosus* (3 Cys, 1 Asp) (*10, 11*) and histidine ligation in Rieske protein (2 Cys, 2 His) (*12*) and three types of hydrogenases (3 Cys, 1 His) (*7, 8, 13, 14*). In addition to the natural occurrence of Fe-S clusters, modified ligands have been produced using site-directed mutagenesis involving serine coordination to Fe-S clusters (*5* and references therein). The spatial configuration of these amino acids in the proper polypeptidic environment seems to determine the type and properties of Fe-S clusters assembled in the proteins.

Little is known about how Fe-S clusters are constructed in polypeptides during the biogenesis of this class of proteins. In *Azotobacter vinelandii*, it has been demonstrated that NifS and NifU are involved in the synthesis of the Fe-S clusters of nitrogenase (*15*). NifS is a pyridoxal phosphate-dependent cysteine desulfurase that decomposes cysteine to alanine and elemental sulfur (*16, 17*). The sulfur is transiently bound to a specific cysteine side chain of the enzyme and then transferred into the Fe-S clusters by an unknown mechanism. NifU is a homodimer that contains one [2Fe-2S] cluster per subunit (*18*). It has been suggested that NifU might function either to deliver iron or to provide an intermediate site for Fe-S cluster assembly, although no experimental evidence is available.

Recently, Zheng and coworkers identified a gene cluster composed of nine genes, ORF1-ORF2-*iscS* (*nifS*-like gene)-*iscU* (*nifU*-like gene)-*iscA-hscB-hscA* (*dnaK*-like gene)-*fdx* ([2Fe-2S] Fd)-ORF3, which is conserved in *A. vinelandii*, *Escherichia coli*, and *Haemophilus influenzae* (19). The occurrence of *nif*-like genes in non-nitrogen fixing organisms led them to propose that these might play a role in the formation or repair of Fe-S proteins and to designate them as *isc* (iron-sulfur cluster) genes. A similar arrangement of the gene cluster is found in the DNA sequence data from several bacteria, including *Yersinia pestis* (http://www.sanger.ac.uk/Projects/Y_pestis/), *Actinobacillus actinomycetemcomitans* (<http://www.genome.ou.edu/act.html>), *Pseudomonas aeruginosa* (<http://www.pseudomonas.com/>), and *Bordetella pertussis* (http://www.sanger.ac.uk/Projects/B_pertussis/), and it appears likely that most of the genes are cotranscribed and encode proteins with coupled functions. This view is supported in part by recent studies on the suppressor mutants of superoxide dismutase (*SOD1*) deficiency in *Saccharomyces cerevisiae*, which revealed that mutations in yeast homologs of IscS (Nfs1p), HscA (Ssq1p), and HscB (Jac1p) result in a severe reduction in mitochondrial Fe-S proteins such as aconitase and succinate dehydrogenase (20).

In *E. coli*, we have been studying the synthesis of Fe-S clusters by overexpressing recombinant Fd as a reporter of protein-bound Fe-S clusters. A previous study has shown that the coexpression of the *isc* gene cluster from a compatible plasmid dramatically increases the production of all five reporter holoFds examined (21). The correct assembly of the Fe-S clusters on respective polypeptides was verified by the absorption spectra of purified Fds. Thus, the capacity of the endogenous system responsible for the synthesis and insertion of Fe-S clusters should be accelerated by the increased expression of the *isc* gene cluster. Intriguingly, the overproduction was dependent on neither the polypeptides of the reporter Fds nor on the type of the Fe-S clusters they contain. These results led us to conclude that the products of the *isc* gene cluster are involved in the assembly of the Fe-S clusters in a wide variety of Fe-S proteins in *E. coli* cells. In the present study, we continue the characterization of the *isc* gene cluster focusing primarily on identifying the genes required for Fe-S cluster formation. We constructed plasmids in which eight ORFs were individually inactivated. By coexpressing these plasmids with reporter Fds, we assigned several genes that are required for the assembly of Fe-S clusters. Also, we examined coexpression using the *fdx* gene as a reporter Fd. Here we demonstrate that the *fdx* gene product (EcFdx) encoded in the *isc* gene cluster has a specific function, probably providing an intermediate site for Fe-S cluster assembly.

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MATERIALS AND METHODS

Materials

E. coli C41(DE3) were provided by J.E. Walker. Enzymes for DNA manipulation were obtained from New England Biolabs, Inc. or TaKaRa Shuzo. Bacterial growth media components were from Difco Laboratories, and other reagents were from Sigma Chemical, Wako Pure Chemical, or Nacalai Tesque.

Construction of Plasmids

Recombinant DNA techniques were carried out according to the established procedures (22). The plasmids for the expression of reporter Fds (pET21-PbPetF, pET21-PbFdxH, pET21-EcFdxN, pET21-RcFdII, and pET21-EcFdx) were constructed using the pET-21a(+) or pET-21d(+) vector as

already described (21). The entire *isc* gene cluster was coexpressed from the pRKISC plasmid, which contains a compatible replication origin (IncP1), and the 7.6-kb *Bst*1107I-*Eco*RI fragment subcloned from Kohara clone #430 (21).

Inactivation of ORF2, *iscS*, *iscA*, *hscB*, *hscA*, or *fdx* in the pRKISC plasmid was carried out by introducing an oligonucleotide linker (5'-TTAATTAATTAA-3'; *Pac*I linker), which contains stop codons (TAA) in all six reading frames, into the respective coding regions. To inactivate ORF2, the pRKISC plasmid was digested with *Kpn*I, a unique site located in the coding region of ORF2, blunt-ended with T4 DNA polymerase, and ligated with the *Pac*I linker to make the pISC-2T plasmid. Inactivation of other genes involved subcloning steps into another vector from which several restriction sites were eliminated. For this purpose, a new vector, pUCKM3, was constructed as follows. A 1.45-kb *Ahd*I-*Bgl*II fragment containing the replication origin and polycloning sites was obtained from pUC119. End-repair of this fragment and ligation with a 1.3-kb kanamycin-resistant cartridge (*Hind*III-*Sma*I fragment, end-repaired) from pMC19 (23) gave pUCKM3. The 7.6-kb *Xba*I-*Nhe*I fragment containing the entire ORF1-ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 gene cluster was excised from the pRKISC plasmid and cloned into an *Xba*I site of pUCKM3 to make the pUCKMISC plasmid. To introduce stop codons in the coding region of *hscA*, the pUCKMISC plasmid was digested with *Hpa*I, a unique site located in the coding region of *hscA*, and ligated with the *Pac*I linker. The resulting plasmid was designated pUCKMISC-hAT. Similarly, pUCKMISC was digested with *Ahd*I or *Sac*II, blunt-ended, and ligated with the *Pac*I linker to make the pUCKMISC-ST or pUCKMISC-AT plasmid, respectively, in which the *iscS* or *iscA* gene was inactivated by the insertion of a stop codon. The 5.4-kb *Eco*RI fragment containing the *iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 gene cluster was excised from Kohara clone #430 and cloned into an *Eco*RI site of pUCKM3 to make the pUCKME plasmid. To inactivate the *fdx* gene, pUCKME was digested with *Bgl*II, blunt-ended, and ligated with the *Pac*I linker to make the pUCKME-FT plasmid. Similarly, the pUCKME plasmid was digested with *Ssp*I and ligated with the *Pac*I linker to make the pUCKME-hBT plasmid, in which the *hscB* gene was inactivated. The modified gene clusters were then transferred to the IncP1-type vector. The 7.6-kb *Xba*I-*Xho*I fragments were excised from the pUCKMISC-ST, pUCKMISC-AT, and pUCKMISC-hAT plasmids and cloned into the *Xba*I/*Xho*I sites of the pRKNMC vector (21) to make the pISC-ST, pISC-AT, and pISC-hAT plasmids, respectively. The 5.4-kb *Eco*RI fragments were excised from the pUCKME-FT and pUCKME-hBT plasmids and cloned into an *Eco*RI site of the pBsE40 plasmid (21) to make the pISC-FT and pISC-hBT plasmids, respectively. The correct orientation was checked by digestion with several restriction enzymes.

Inactivation of ORF1 or ORF3 in the pRKISC plasmid was carried out by truncating the respective coding regions. The 1.4-kb *Nco*I-*Eco*RI fragment (the *Nco*I site was end-repaired) containing truncated ORF1 and intact ORF2 was excised from the pRKISC plasmid, and inserted into the pRKNSE vector (21), which had been cut with *Kpn*I, blunt-ended, and digested with *Eco*RI. The resulting plasmid (pNcE32) was digested with *Eco*RI and ligated with the 5.4-kb *Eco*RI fragment containing the *iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 gene cluster excised from Kohara clone #430. The correct orientation was verified by digestion with several restriction enzymes and the plasmid was designated pISCΔ1. The 5.0-kb fragment containing the *iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx* cluster and truncated ORF3 was excised from the pUCKME plasmid, which had been digested with *Nsi*I, blunt-ended, and digested with *Eco*RI. The fragment was cloned into the pBsE40 plasmid (21), which was digested with *Xho*I, blunt-ended, and digested with *Eco*RI. The resulting plasmid was designated pISCΔ3. Similarly, a 4.6-kb *Eco*RI-*Bgl*II fragment (the *Bgl*II site was end-repaired) containing the *iscS*-*iscU*-*iscA*-*hscB*-*hscA* cluster and truncated *fdx* gene was excised from pUCKME and cloned into the *Eco*RI/*Xho*I sites (*Xho*I site was end-repaired) of pBsE40. The resulting plasmid was designated pISCΔF3.

Expression and Determination of holoFds

The competent *E. coli* strain C41(DE3) (24) was transformed either with pET21-PbPetF, pET21-PbFdxH, pET21-EcFdxN, pET21-RcFdII, or pET21-EcFdx. For coexpression, cells harboring one of the pET21-derived plasmids were transformed with pRKISC or the modified plasmids described above. The pRKNCM vector was also introduced into the cells for control experiments. The bacteria were cultivated in 4 ml of Terrific broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline to an absorbance of approximately 0.5 at 600 nm, and the production of Fd was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). In some experiments, ferric ammonium citrate was added to the culture medium at a concentration of 0.1 mg/ml. The cells were grown aerobically for 18 h at 28°C, pelleted, and suspended in a solution containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, and 0.1 mg/ml lysozyme at a cell density represented by $A_{600} = 30$. After incubation at 30°C for 5 min, the cells were disrupted by sonication. The suspension thus obtained was centrifuged at 15,000 x g for 15 min at 4°C.

Fds were determined from the *E. coli* extracts essentially as described elsewhere (21) with some modifications. The supernatant fraction was diluted with a solution containing 25 mM Tris-HCl, pH 7.5, and 50 mM NaCl, filtered through 0.1 µm Durapore membrane (Ultrafree-MC; Millipore), and applied to a DEAE-5PW column (2 x 7.5 mm, Tosoh) equilibrated with 25 mM Tris-HCl, pH 7.5, and 50 mM NaCl. The column was developed at a flow rate of 0.1 ml/min with a 20-min linear gradient from 50 to 540 mM NaCl using an HPLC system (Nanospace SI-1; Shiseido). The elution of Fd was monitored by recording the absorbance at 400 nm and quantified by peak integration using purified Fds as standards. Chromatography on the semimicro column was more sensitive than that previously described (21), and permitted the quantification of Fd amount as small as 50 ng with reproducible results.

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RESULTS AND DISCUSSION

Identification of Genes Involved in the Assembly of Fe-S Clusters in Reporter Fds

The *isc* gene cluster is encoded on the complementary strand at bases 2661343-2654556 (57.3-57.2 min) of the *E. coli* genome sequence (25). The cluster contains nine ORFs: ORF1 (b2532), ORF2 (b2531), *iscS* (formerly *yyzO* or b2530), *iscU* (b2529), *iscA* (*yfhF*), *hscB* (*yfhE*), *hscA*, *fdx*, and ORF3 (*yfhJ*) (Fig. 1). When the *isc* gene cluster was subcloned into a plasmid (pRKISC) and coexpressed with reporter Fds from compatible plasmids, the assembly of the Fe-S clusters was dramatically accelerated, leading to the overproduction of reporter holoFds (21). In order to identify the genes responsible for Fe-S cluster formation, the pRKISC plasmid was modified as shown in Fig. 1. Inactivation of the individual ORFs was carried out either by truncating the coding regions or by introducing an oligonucleotide linker (TTAATTAATTAA). The linker contains stop codons (TAA) in all six reading frames along with *PacI* and *Asel* restriction sites. The intact and modified gene clusters were inserted into the IncP1-type vector (pRKNCM) in the same direction as the *lac* promoter. Inactivation of the *iscU* gene could not be achieved due to the lack of an appropriate restriction site in the coding region.

The eight modified plasmids were coexpressed with the pET21-PbPetF (encoding

cyanobacterial [2Fe-2S] PetF), pET21-PbFdxH (cyanobacterial [2Fe-2S] FdxH), pET21-EcFdxN (2[4Fe-4S] FdxN from *E. coli*), or pET21-RcFdII ([3Fe-4S][4Fe-4S] FdII from *R. capsulatus*) plasmid in *E. coli* strain C41(DE3) and examined for their ability to promote the overproduction of the reporter holoFds. In these experiments, *E. coli* cells containing two coexpression plasmids were grown in Terrific broth supplemented with 0.1 mg/ml ferric ammonium citrate and 1 mM IPTG. Reporter holoFds produced in the cells were determined from the bacterial lysates by an anion-exchange HPLC system monitoring the absorbance of the effluent at 400 nm. As shown in Fig. 2, truncation of ORF1 and ORF3 in the coexpression plasmid (the pISC Δ 1 and pISC Δ 3 plasmids, respectively) did not affect the overproduction of any Fds. In these cells, the amount of reporter holo-PbPetF, -PbFdxH, -EcFdxN, and -RcFdII was the same within experimental error as that determined in control cells carrying the pRKISC plasmid. In contrast, inactivation of the *iscS* gene in the coexpression plasmid (the pISC-ST plasmid) had a severe effect on the production of all reporter Fds. The low level of production was similar to or below that of the negative control (the pRKNMC vector). Similarly, inactivation of the *iscA*, *hscA*, and *fdx* genes in the plasmid-encoded *isc* gene cluster (pISC-AT, pISC-hAT, and pISC-FT, respectively) reduced the production of all the reporter holoFds examined. When the *hscB* gene was inactivated (pISC-hBT), the productions of holo-PbPetF, -EcFdxN, and -RcFdII were slightly reduced, while no clear decrease in holo-PbFdxH was observed. As for the inactivation of ORF2 (pISC-2T), decreased production was appreciable only in the case of holo-PbPetF. These results indicate that coexpression of the *iscS*, *iscA*, *hscA*, and *fdx* genes is a prerequisite for the overproduction of all reporter holoFds.

Genes Required for the Assembly of the [2Fe-2S] Cluster in the *fdx* Gene Product (EcFdx)

The *fdx* gene in the *isc* gene cluster codes for [2Fe-2S] Fd (EcFdx) (21, 26, 27). The finding that EcFdx is involved in the assembly of Fe-S clusters in other reporter Fds prompted us to examine the effect of the coexpression of the *isc* gene cluster on the production of this protein. When the *fdx* gene was expressed from the pRKISC plasmid, however, the production of EcFdx was limited and could not be clearly detected (21). Therefore, the protein was expressed as a reporter Fd using the pET21-EcFdx plasmid. As shown in Fig. 3a, holo-EcFdx production was increased 2.5-fold by coexpression with the intact *isc* gene cluster (pRKISC). Not surprisingly, this overproduction was not affected by the inactivation of the *fdx* gene in the coexpression plasmid (pISC-FT). Inactivation of *iscS* (pISC-ST), *hscB* (pISC-hBT), and *hscA* (pISC-hAT) resulted in a reduced production of holo-EcFdx in a manner similar to that observed with other reporter Fds. In contrast, inactivation of the *iscA* gene (pISC-AT) did not affect the overproduction. When ORF3 was truncated (pISC Δ 3), a small but significant decrease was observed compared to the coexpression with the intact *isc* gene cluster (pRKISC). The involvement of ORF3 was further confirmed by coexpression with the pISC Δ F3 plasmid, in which ORF3 and most of the coding region of *fdx* were removed from the plasmid. These results indicate that the *iscS*, *hscB*, *hscA*, and ORF3 gene products are involved in the assembly of the [2Fe-2S] cluster in EcFdx.

We have previously observed that the production of holoFds in *E. coli* varies depending on the culture medium. In particular, the overproduction of holo-PbPetF, -PbFdxH, and -EcFdxN by coexpression with the *isc* gene cluster was further increased by about twofold by supplementing the Terrific broth with iron (21). Thus, the modified gene cluster was examined both in the presence and absence of supplemental iron with the hope that some mutation might provide a clue as to the component involved in the utilization of iron. In the absence of

supplemental iron, the effect of inactivation on the production of holo-PbPetF and -EcFdxN was virtually the same as that observed in the presence of excess iron, except that the level of production was only about one-half (not shown). In contrast, a small but significant difference could be seen in the production of holo-EcFdx, which was not affected by the truncation of ORF3 in the plasmid-encoded *isc* gene cluster (pISC Δ 3 and pISC Δ F3) when the culture medium was not supplemented with iron (Fig. 3b). Because the inactivation of *iscS* (pISC-ST), *hscB* (pISC-hBT), and *hscA* (pISC-hAT) produce severe effects on holo-EcFdx production in both the presence and absence of excess iron, the ORF3 product might be functionally related to iron utilization.

The Role of EcFdx in the Assembly of Fe-S Clusters

We have shown that several proteins encoded by the *isc* gene cluster are required for the assembly of Fe-S clusters. The most interesting result of this study is the identification of the involvement of EcFdx. The EcFdx protein shares 29% amino acid identity with human mitochondrial adrenodoxin and putidaredoxin from *Pseudomonas putida* (Fig. 4a). The purified EcFdx has one [2Fe-2S] cluster (21, 26-28), which should be coordinated by four cysteine residues in the conserved CxxxxxCxxC//C sequence motif. Thus, the Fe-S protein should be involved in the assembly of the Fe-S clusters of other proteins. To do this, EcFdx might have a redox function, since the related Fds, adrenodoxin and putidaredoxin, function in electron transfer to cytochrome P450 enzymes. A low midpoint potential (-0.38 V) of EcFdx (26) would facilitate the reduction of Fe^{3+} to Fe^{2+} and S^0 to S^{2-} , if these reduced compounds are required for the Fe-S cluster assembly. However, the physiological electron donor and acceptor for EcFdx have not been unequivocally identified (27). It also seems unlikely that the redox partner is encoded in the *isc* gene cluster as judged from their deduced amino acid sequences.

Another possibility is that the EcFdx protein functions to provide an intermediate site in the assembly of Fe-S clusters. To support this view, the amino acid sequences of EcFdx and bacterial homologs are rich in conserved cysteine and histidine residues (Fig. 4a). As only four cysteines are required to coordinate the [2Fe-2S] cluster, there are two other cysteine and three histidine residues which have the potential to coordinate additional iron atoms. Also, the thiolate side chains of cysteine residues can bind additional sulfur atoms in the form of cysteine persulfide. It is worth noting that some of these residues are located in close vicinity to the CxxxxxCxxC sequence, which should be well suited for receiving iron and/or sulfur to build up the [2Fe-2S] cluster and convert it to the [3Fe-4S] or [4Fe-4S] form. The latter consideration was prompted by a previous observation that the [2Fe-2S] cluster of *E. coli* biotin synthase is reversibly converted to a [4Fe-4S] cluster via dimerization (29). Similarly, the [4Fe-4S] clusters of FNR protein (fumarate nitrate reduction; O_2 sensor for anaerobic growth) and nitrogenase Fe-protein are converted to the [2Fe-2S] form upon exposure to oxygen or chelators (30-32). The facile interconversion of [3Fe-4S]/[4Fe-4S] clusters has been demonstrated in *Desulfovibrio gigas* Fd II (33), *D. africanus* Fd III (34), *P. furiosus* Fd (35), and aconitase (3, 4), in which the [3Fe-4S] cluster can reversibly incorporate an additional Fe to form a [4Fe-4S] cluster. In addition to the protein-bound Fe-S clusters, numerous cases are recorded of the interconversion of synthetic Fe-S clusters liganded by aryl thiolates or alkyl thiolate (4). In the biosynthesis of Fe-S clusters, the [2Fe-2S] cluster of the EcFdx protein might be converted to the [4Fe-4S] and [3Fe-4S] forms on the cysteine- and histidine-rich scaffold prior to transfer to other proteins such as EcFdxN and RcFdII.

If EcFdx does bind additional iron and/or sulfur atoms destined for Fe-S cluster assembly and

conversion, one would anticipate that such elements would be associated with the isolated protein. Actually, all of the irons and inorganic sulfides in isolated EcFdx can be accounted for by a [2Fe-2S] cluster (28). However, our preliminary study on purified EcFdx indicates that the protein has been converted to another molecular species upon incubation with S^{2-} and Fe^{2+} or Fe^{3+} . The molecule was clearly separated from EcFdx (as isolated) by anion-exchange chromatography, but was unstable and reverted to the original [2Fe-2S] form within a few hours (not shown). The conversion is a specific reaction observed with EcFdx and not with other Fds including PbPetF, PbFdxH, EcFdxN, and RcFdlI, indicating an unusual function of EcFdx. In the *in vivo* reaction, the additional iron and sulfur atoms bound to EcFdx should be released during formation of the Fe-S cluster and would be loosely associated with the polypeptide and, therefore, readily lost during purification. The IscA-independent formation of the [2Fe-2S] cluster of EcFdx shown in this study provides additional evidence that EcFdx might function prior to IscA, probably as an intermediate site in the assembly of Fe-S clusters for other Fds (discussed below).

Proposed Scheme for the Assembly of Fe-S Clusters

Based on the present study, we propose the following model. The overall reaction responsible for the Fe-S cluster formation can be divided into two sequential steps; assembly of the Fe-S cluster in the *fdx* gene product (EcFdx) as an intermediate and its transfer to other proteins (Fig. 5). Four proteins encoded by *iscS*, *hscB*, *hscA*, and ORF3 are involved in the first step (Fig. 3). The IscS protein is a homolog of NifS, and pyridoxal phosphate-dependent cysteine desulfurase activity has been demonstrated for both proteins (16, 17, 36). Thus, IscS is the donor of sulfur atoms destined for the Fe-S cluster assembly. There are two additional genes contained within the *E. coli* genome that are homologous to *iscS*. One has been cloned and characterized as a cysteine sulfinatase with selenocysteine lyase and cysteine desulfurase activities (37). The second *E. coli* gene of this family (*sufS*) is located within the *sufABCDSE* gene cluster. The physiological function of the gene cluster is not clear, but seems to be related to the assembly of the [2Fe-2S] cluster of the FhuF protein (38). Interestingly, the product of the first gene, *sufA*, is similar to IscA.

ORF3 codes for a small protein of 66 amino acids with a pI value of 3.7, which is involved in the assembly of the [2Fe-2S] cluster in EcFdx but not clearly in other reporter Fds. Furthermore, the effect of ORF3 inactivation is appreciable only in the presence of supplemental iron in the culture medium (Fig. 3). Based on a situation in which the genomic copy of the *isc* gene cluster is intact, these results indicate that the ORF3 product is required in larger amounts for full overproduction of holo-EcFdx in the presence of excess iron. We believe that the protein has a function, perhaps binding iron atoms and transferring them to EcFdx. Given the very low concentration of free iron in the cell, it might be that an iron-specific 'metallochaperone' is involved in the reaction. The highly acidic nature of the ORF3 protein rich in aspartic acid and glutamic acid residues is conserved in bacterial homologs (Fig. 4c), and this might facilitate an ionic interaction with Fe^{2+} and/or Fe^{3+} . In addition, the carboxylate groups of aspartic acid and glutamic acid are known to act as ligands for iron in several Fe-containing proteins including transferrin, myohemerythrin, ribonucleotide reductase, and ferritin (39, 40).

The HscA protein (Hsc66) is an Hsp70 chaperone and shares 41% sequence identity with *E. coli* DnaK (41). It has been demonstrated that HscA is unable to replace DnaK in the chaperone-assisted refolding of unfolded peptides either *in vivo* or *in vitro*, and suggesting that the peptide substrate specificity of HscA is different from that of DnaK (42-44). The HscB

protein (Hsc20) appears homologous to a subfamily of J-domain containing co-chaperones (42, 45). *In vitro* studies have shown that HscB elevates the ATPase activity of HscA by several fold but not the activity of DnaK (42, 43). Therefore, HscB functions as a specific co-chaperone that regulates the ATP-dependent activity of HscA. The HscA function is necessary for the overproduction of all reporter holoFds examined in this study (Figs. 2 and 3). The involvement of HscB is less clear than that of HscA, but significant in the case of holo-PbPetF, -EcFdxN, -RcFdxII, and -EcFdx production. These heat-shock-cognate proteins might serve as molecular chaperones either by stabilizing the unfolded state of apoFd polypeptides, or by assisting the folding reaction after the assembly of Fe-S clusters. A role in destabilizing the structure of holo-EcFdx is also possible, and this may assist the extrusion of the preassembled Fe-S cluster from the EcFdx polypeptide for transfer to other proteins. Interestingly, the ATP-dependent formation of the [2Fe-2S] cluster of Fd has previously been demonstrated in both intact chloroplasts and their extracts (46-48). Also, mutations in the yeast homologs of HscA (Ssq1p) and HscB (Jac1p) result in a sharp decrease in mitochondrial Fe-S proteins, such as aconitase and succinate dehydrogenase, in a manner similar to the mutation of the *IscS* homolog (Nfs1p) (20). These observations indicate the general roles of the chaperone-mediated assembly of Fe-S clusters in a wide variety of organisms.

The *iscA* gene product is necessary for the overproduction of holo-PbPetF, -PbFdxH, -EcFdxN, and -RcFdxII, but not for the assembly of the [2Fe-2S] cluster in EcFdx (Figs. 2 and 3). Our results strongly support the notion that *IscA* is involved in the transfer of the preassembled Fe-S cluster from EcFdx to other Fds. A search of the data banks revealed that proteins homologous to *IscA* are widely distributed not only in bacteria but also in yeast, animals, and plants in a manner similar to the *IscS*, *IscU*, and *HscA* homologs. There is a strong conservation of three cysteines at residues 35, 99, and 101 of *E. coli* *IscA* (19), which might be involved in the transient coordination to the Fe-S cluster during the transfer process. As for the ORF2 product, it is premature to draw a firm conclusion about its role since an effect of its inactivation was observed only in the case of holo-PbPetF production (Fig. 2). However, it is tempting to speculate that it functions as another carrier of Fe-S clusters since the conserved cysteine and histidine residues are arranged in a CxxxxxCxxxxCxxH sequence (Fig. 4b).

With regard to the ORF1 product, we have no evidence for its role. Truncation of ORF1 in the coexpression plasmid did not lead to a defect in the overproduction of any holoFds. ORF1 is separated from ORF2 by 455 nucleotides and a large hairpin structure composed of 215 nucleotides lies in the intergenic region. Hexanucleotide sequences TTGACC and CAGACT upstream of ORF2 at positions -104 to -99 and -81 to -76 show sequences and spacing similar to the -35 and -10 promoter consensus sequences (25). Thus, ORF1 is likely to be contained in a transcription unit that is separate from the ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 cluster. Since separate transcription units have also been demonstrated in *A. vinelandii* (19), the ORF1 product does not appear to play a role in the coupled functions of other proteins described here. As for the *iscU* gene, we could not examine its role due to the lack of an appropriate restriction site in the coding region. The wide distribution of *IscU* homologs and the conservation of three cysteine residues have been demonstrated elsewhere (19). The NifU protein is also a homolog of *IscU*. NifU exists as a homodimer containing one [2Fe-2S] cluster per subunit (18). Although the specific function of NifU remains unknown, its activity is required for the full activation of the nitrogenase component proteins (15). It should be noted that the *IscU* protein is a truncated form of NifU and the sequence similarity is restricted to the N-terminal domain of NifU. In the middle domain of NifU, four cysteine residues arranged in a CxC//CxC sequence are involved in the coordination to the [2Fe-2S] cluster (19), and in the C-terminal domain, an additional CxC

motif is found in NifU and its homologs (49). In view of the fact that the NifU protein has a chimeric structure composed of three domains, the middle and C-terminal domain might function as an intermediate site in the assembly of Fe-S clusters in a manner similar to the EcFdx protein shown in this study. If this is the case, the conserved structure in the IscU protein and the N-terminal domain of NifU might be indicative of a crucial role either in building the [2Fe-2S] cluster, or in its conversion to the [4Fe-4S] and [3Fe-4S] clusters, or both.

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CONCLUSION

The biological formation of Fe-S clusters is a complex reaction requiring several gene products. The present study points to the possibility that proteins encoded by the ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster play important roles in the assembly of Fe-S clusters. These findings are consistent with a recent report that the yeast homologs of IscS (Nfs1p), HscA (Ssq1p), and HscB (Jac1p) are involved in the maturation of mitochondrial Fe-S proteins such as aconitase and succinate dehydrogenase (20, 50). In particular, it is of interest to note that EcFdx participates in the reaction probably by providing an intermediate site for Fe-S cluster assembly. Further studies involving amino acid substitutions and disruption of the genomic copy should provide us more information about the proposed mechanism. Also, because large amounts of reporter Fds are now available, and the genes involved in the reaction have been identified, it is possible to initiate *in vitro* biochemical reconstitution experiments aimed at determining whether or not these proteins participate directly, either separately or in combination, in the distinct steps of Fe-S cluster assembly. We are grateful to Dr. J.E. Walker of the Medical Research Council (UK) for providing the C41(DE3) strain and Dr. H. Mori of the Nara Institute of Science and Technology for providing the Kohara clone #430. We thank Dr. K. Saeki for providing the *PacI* linker and for helpful discussion.

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Abbreviations: EcFdx, *Escherichia coli* ferredoxin encoded by the *fdx* gene; EcFdxN, *E. coli* ferredoxin encoded by the *fdxN* gene; Fd, ferredoxin; IPTG, isopropyl-1-thio- β -D-galactopyranoside; ORF, open reading frame; PbFdxH, *Plectonema boryanum* ferredoxin encoded by the *fdxH* gene; PbPetF, *P. boryanum* ferredoxin encoded by the *petF* gene; RcFdII, *Rhodobacter capsulatus* ferredoxin II encoded by the *fdxA* gene.

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